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Simultaneous Detection of *Escherichia coli*, *Salmonella enterica*, *Listeria monocytogenes* and *Bacillus cereus* by Oligonucleotide Microarray

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Background: Traditional laboratory methods to detect pathogenic bacteria are time consuming and laborious. Therefore, it is essential to use powerful and reliable molecular methods for quick and simultaneous detection of microbial pathogens.
Objectives: The current study aimed to evaluate the capability and efficiency of *23S rDNA* sequence for rapid and simultaneous detection of four important food-borne pathogens by an oligonucleotide microarray technique.
Materials and Methods: The *23S rDNA* sequences of *Escherichia coli*, *Salmonella enterica*, *Listeria monocytogenes* and *Bacillus cereus* were obtained from GenBank databases and used to design the oligonucleotide probes and primers by Vector NTI software. Oligonucleotide probes were placed on a nylon membrane and hybridization was performed between probes and *23S rDNA* digoxigenin-labeled polymerase chain reaction (PCR) products. Hybridization signals were visualized by NBT/BCIP color development.
Results: Positive hybridization color was produced for *Escherichia coli*, *Salmonella enterica*, *Listeria monocytogenes* and *Bacillus cereus*. The oligonucleotide microarray detected all bacterial strains in a single reaction in less than five hours. The sensitivity of the performed microarray assay was 10^3 cfu/mL for each species of pathogen. No cross reaction was found between the tested bacterial species.
Conclusions: The obtained results indicated that amplification of *23S rDNA* gene followed by oligonucleotide microarray hybridization is a rapid and reliable method to identify and discriminate foodborne pathogens tested under the study.

Keywords: Oligonucleotide Microarray, Pathogenic Bacteria, Hybridization, *23S rDNA*

1. Background

Pathogenic bacteria are responsible for life-threatening diseases in humans and pose a serious challenge to public health worldwide (1, 2). The clinical syndromes caused by different food-borne and intestinal pathogens are usually not distinguishable. Therefore, the identification of pathogens greatly depends on the help provided by clinical laboratories (3, 4). There are several traditional microbiological methods to detect pathogenic bacteria, which rely on culture followed by standard biochemical and serological methods (5, 6). These methods are very time consuming, onerous and not sensitive enough. Furthermore, these methods are unable to detect several pathogens simultaneously. Therefore it is essential to use powerful and reliable molecular methods for quick and simultaneous detection of microbial pathogens (7-9). Over the past ten years, several molecular methods were increasingly used to improve the sensitivity and speed of diagnosis in clinical microbiology. Most of them have several advantages over traditional microbiological methods including shorter time needed for data analysis, low detection limits, higher specificity and sensitivity (2, 10, 11). DNA hybridization using oligonucleotide microarray is a specific technique to detect microbial pathogens. In this technique, probes are designed and synthesized for different genes and deposited on glass slides or nylon membranes, or may be directly synthesized on them. The arrays have the ability to perform a series of simultaneous hybridization assays that can be easily interpreted. Hybridization patterns of DNA sequences with specific probes indicate the presence or absence of specific microorganisms in the sample (12-14). DNA microarrays are developed to detect and identify a large number of bacteria present in food, blood, stool and urine (15-17). Miller et al. (18) reported that microarray technology and its application to diagnose infectious diseases, is vastly grew from 2000 to 2008. Among the *rDNA* genes in bacterial genomes, the *23S rDNA* genes are the most frequently used markers and are repeatedly applied to detect and identify intestinal pathogens by DNA microarray (5, 7, 12). Wilson et al. (19) described the multi pathogen identification (MPID) microarray for high confidence identification of eighteen pathogens. Using this detection system, these eighteen pathogens were screened for their presence by

the examination of *rDNA* specific regions. Anthony et al. (16), introduced a rapid detection and identification system that uses universal polymerase chain reaction (PCR) primers to amplify a variable region of *23S rDNA* by reverse hybridization of the products to a panel of oligonucleotides from a wide range of clinically significant bacterial species in blood cultures. Several studies report that *23S rDNA* based microarray using specific sequences for each bacterial species can easily identify and discriminate bacterial pathogens (16, 20, 21).

2. Objectives

The current study aimed to evaluate the capability and efficiency of *23S rDNA* sequences for rapid and simultaneous detection of *Escherichia coli*, *Salmonella enterica*, *Listeria monocytogenes* and *Bacillus cereus* by oligonucleotide microarray.

3. Materials and Methods

3.1. Preparation and Cultivation of Bacterial Strains

Bacterial reference strains used in the current study were *Escherichia coli* (ATCC 11303), *Salmonella* spp. (ATCC 14028), *Listeria monocytogenes* (ATCC 19114) and *Bacillus cereus* (ATCC 11778) provided by the Centers for microbial culture collection (CMCC), Pasteur Institute of Iran. These strains were transferred to brain heart infusion broth (BHI; Sigma Chemical Co., St Louis, MO, USA) and cultivated at 37˚C for 24 - 36 hours. Pure culture of each strain was diluted from 10^6 to 1 cfu/mL. Two mixed samples were prepared as follows: No.1 contained *E. coli*, *S. enterica* and *B. cereus*; No. 2 contained *S. enterica*, *L. monocytogenes* and *B. cereus*. Freshly cultured bacterial samples (10^9 cfu/mL) were serially diluted tenfold to 1 cfu/mL, as determined by colony forming unit (CFU) count, in 0.1 M PBS buffer (pH 7.5). DNA was extracted and amplified by PCR and after that microarray was performed for the lower detection limit of the dilution.

3.2. DNA Extraction and PCR

Bacterial DNA was extracted using the genomic DNA purification kit (Fermentas, Lithuania) according to the manufacturer protocol. Five microliters of the DNA template was amplified by PCR in 25 μL of 1 × PCR buffer containing 200 μmol/L of each dNTP, 2 U Taq DNA polymerase (Fermentas, Lithuania) and 1 μmol/L of each forward and reverse primers. Amplification conditions were one cycle at 94˚C for four minutes, then 35 cycle consisting of denaturation (94˚C for 45 seconds), annealing (60˚C for 45 seconds), and extension (72˚C for 45 seconds), with a final extension step at 72˚C for three minutes. Quality of PCR products Digoxigenin- (DIG) labeling were tested with DIG nucleic acid detection kit (Roche, Mannheim, Germany) according to the manufacturer protocol.

3.3. Primers and Probes

Four bacterial *23S rDNA* sequences were obtained from the GenBank database. The GenBank accession numbers for the *23S rDNA* sequences are provided in Table 1. Alignment of the *23S rDNA* sequences of different species were performed by the Clustal W algorithm with Align X (a component of Vector NTI Advance 11.0) and areas displaying sequence divergence among species were used for probe selection. The selected variable regions of the alignment were checked for self-binding, GC content, secondary structure, and melting temperature (Tm) by the Vector NTI (Invitrogen Corporation, Carlsbad, CA, US) software and screened for homology with other bacterial sequences using National Center for Biotechnology BLAST (Basic Local Alignment Search Tool). To detect *23S rDNA* gene, the primers were designed from the conservation region of the two ends of this gene fragment as follows: 23S-F 5'-ACCAGGATTTTGGCTTAGAAG-3' (corresponding to *E. coli* 1051 - 1071 nucleotide sequence of *23S rDNA* gene) and 23S-R 5'-digoxigenin-CACTTACCCCGACAAGGAAT-3' (corresponding to *E. coli* 1938 - 957 nucleotide sequence of *23S rDNA* gene) (11). The forward primer was labeled with a digoxigenin dye (Metabion, Germany).

3.4. Preparation of the Oligonucleotide Array

The probes listed in Table 1 were suspended at a concentration of 20 μmol/L, and 1 μL of each probe was spotted at a specific position of the SensiBlot Plus Nylon Membrane (Fermentas, Lithuania). To facilitate the hybridization analysis of the different bacterial species, the 12 oligonucleotide probes were arrayed in suitable grids on the nylon membrane (Table 1). The oligonucleotide probes were fixed on the membrane, and cross linked by UV crosslinker for 30 seconds to allow binding of probes onto the nylon membrane. After cross linking, any unbounded oligonucleotides were removed by two times washing in 0.5x SSC, 0.1% sodium dodecyl sulfate (SDS) for two minutes at 37˚C (5, 11). The strips were dried and stored at room temperature. The layout of the probes is listed in Figure 1. The numbers in the layout are listed in Table 1.

3.5. Hybridization

All reagents except buffers were included in the DIG nucleic acid detection kit (Roche, Mannheim, Germany). In brief, each coated membrane was pre-hybridized for one hour with 1 mL of hybridization solution (5x SSC, 0.2% SDS, 0.1% blocking reagent in a petri dish. 10 μL of DIG-labeled PCR products were denatured by heating at 95˚C for five minutes and quick cooling in an ice bath. Then the denatured PCR product was diluted with 0.5 mL of hybridization solution and added to the pre-hybridized membrane. Hybridization was carried out at 50˚C for 60 minutes with gentle shaking. The membranes were washed twice in 1

mL of washing buffer (2x SSC, 0.1% SDS) for five minutes. Approximately 0.5 mL of alkaline phosphatase conjugated sheep anti-digoxigenin antibodies (diluted 1:5000 in blocking solution) was added and the plate was incubated at 37˚C for one hour. NBT/BCIP color development was clearly visible between 30 minutes and one hour after the start of the reaction (11, 13).

4. Results

4.1. Amplification of 23S rDNA Gene Fragment

Universal primers on the basis of the previously described conserved regions of the bacterial *23S rDNA* were successful to amplify the region of interest. The PCR products showed bands at approximately 900 base pair, as expected (Figure 2). Quality control of PCR products DIG labeling showed that all of them were labeled with DIG.

4.2. Hybridization Results for the Pure Bacterial Culture and Mock Samples

PCR products from each bacterial sample were hybridized with oligonucleotide probes attached to nylon membranes. The results showed high specificity of hybridization with the four bacterial species under investigation (Figures 3A - D). Hybridization results of mock samples including bacterial species of *S. enterica*, *E. coli*, and *B. cereus* (Sample 1), and S. enterica, *L. monocytogenes*, and *B. cereus* (Sample 2) are shown in Figures 3E and 3F.

4.3. Sensitivity of Oligonucleotide Microarray

PCR assays were randomly performed to test the lower detection limit of the DNA microarrays. Positive signal could be obtained from dilutions between 10^9 and 10^3 cfu/mL. The results showed that the sensitivity of oligonucleotide microarray was 10^3 cfu/mL.

Table 1. Oligonucleotide Probes Used in the Study

Probe No.	Target	GenBank Access No.	Sequence (5' to 3')	References
1	Shuffled GFP [a] (negative control)	-	CAGCGAGTGTGATATGAGTGATGAGG	(13)
2	*Eschrichia coli*	V00331	CTGATATGTAGGTGAGGTCCCT	(11)
3	*Eschrichia coli*	AJ278710	CTGATATGTAGGTGAAGCGACTTGC	This study
4	*Escherichia coli*	AF053968	CACGCTGATATGTAGGTGAAGTCCC	This study
5	*Salmonella enterica*	U77919	AAATCCGGTTCACTTTAACACTGAGGCGTG	(11)
6	*Salmonella* spp.	U77919	GAAGTGATTTACTCATGGAGCTGAAGTC	This study
7	*Salmonella* spp.	AL627282	TGAAGTCAGCCGAAGATACCAGC	This study
8	Enterbacteriaceae	V00331	GATGTAACGGGGCTAAACCA	(11)
9	*Listeria monocytogenes*	X64533	CGTCCAAGCAGTGAGTGTGAGAAGT	This study
10	*Bacillus cereus*	X94448	GTGCTGGAAGGTTAAGGAGAGGG	This study
11	Bacterial universal probe	V00331	ACGGTCCTAAGGTAGCGAAA	(11)
12	Aequorea victoria GFP [b] (positive control)	AB103336	CAGAGTGTGCGATATTGATGAAAGTG	(13)

[a] Shuffled GFP that contains no sequence similarity to the known genes in GenBank database.
[b] Green Fluorescent Protein.

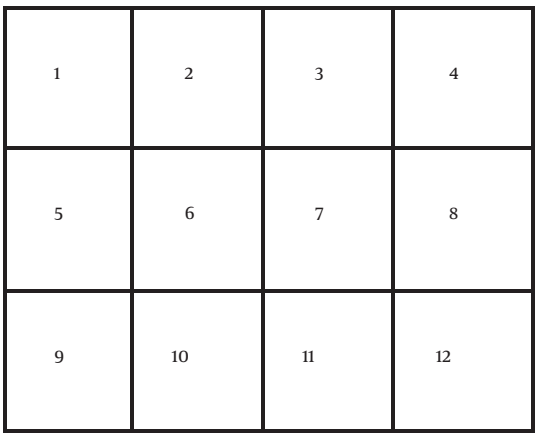
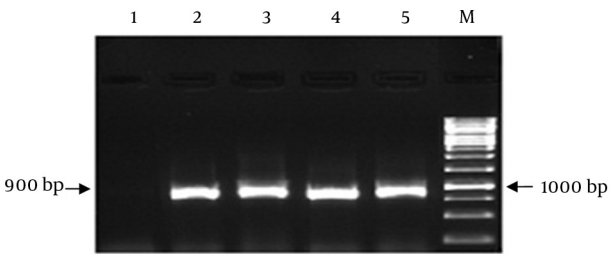


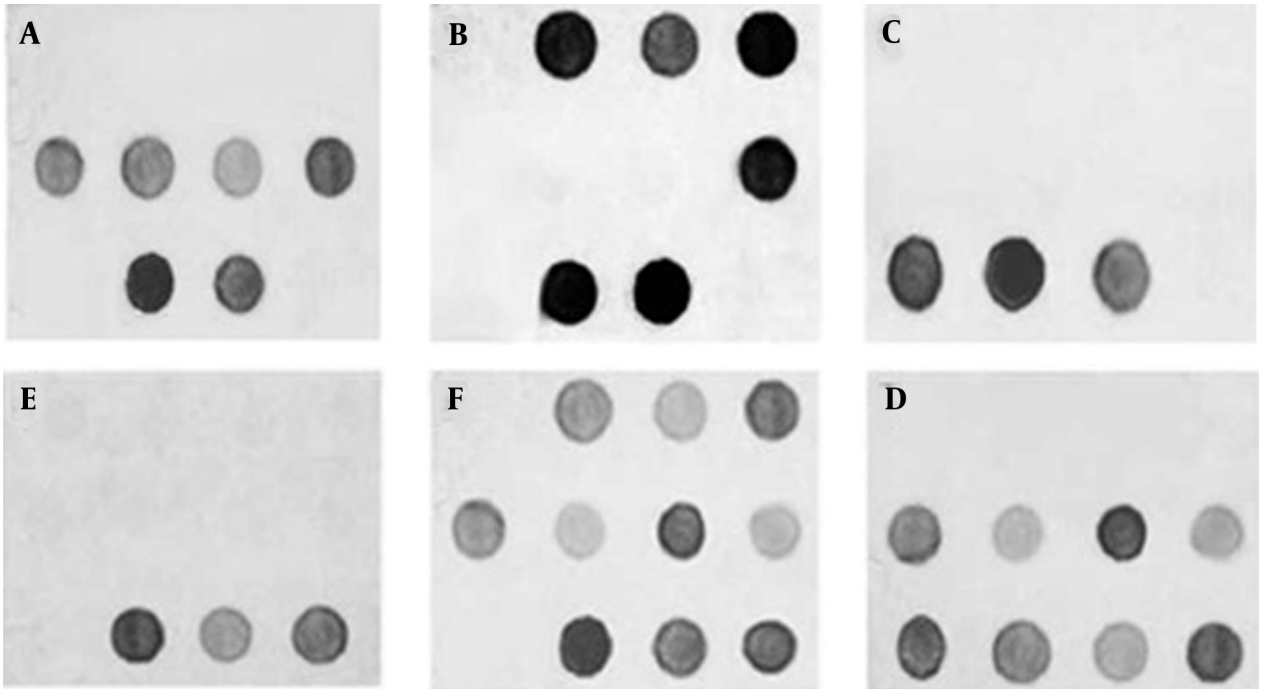
Figure 1. Layout of the Oligonucleotide Probes for *23S rDNA*

Figure 2. Amplification of *23S ribosomal DNA* Gene from the DNA of Four Bacterial Species



Lane 1, the negative control; Lanes 2 - 5, PCR products from *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enterica*, and *Bacillus cereus*; Lane M, 1000 bp molecular size DNA marker.

Figure 3. Oligonucleotide Hybridization Results of Four Species Pathogenic Bacteria



A, *Salmonella enterica*; B, *Escherichia coli*; C, *Listeria monocytogenes*; D, *Bacillus cereus*; E and F are hybridization results of two mock samples; E, *E. coli*, *S. enterica* and *B. cereus*; F, *S. enterica*, *L. monocytogenes* and *B. cereus*.

5. Discussion

From the late 20th century till now, bacterial infectious diseases are remarkably responsible for morbidity and mortality in both humans and animals (1, 10, 22). Routine detection of bacterial pathogens in clinical laboratories is commonly based on microscopic observation, enrichment culture and biochemical and serological methods. All these routine techniques have several limitations such as long culture time, reliance on enrichment and selective culture, and difficulty of quantitative analysis (23, 24).

Oligonucleotide microarray coupled with PCR can serve to enhance rapidity and effectiveness of microbial detection and identification (22-25). Selecting proper target genes is very critical in oligonucleotide microarray methods (14). Various target regions are used to identify pathogens in oligonucleotide microarray methods. These regions include *16S rDNA*, *23S rDNA* and *16S-23S rDNA* internal transcribed spacer region (ITS) (14, 16, 26, 27). The *23S rDNA* genes appear to be the best performing regions as target sequences. The *23S rDNA* has highly specific sequences and can be used easily as a marker to differentiate bacterial pathogens. Recently, there have been several reports on application of *23S rDNA* to identify bacterial species (16, 20, 21). Anthony et al. (16) reported that the *23S rDNA* genes show more variation between species of medically important than the *16S rDNA* genes. They concluded that the accuracy and discriminating power of the assay can be continually extended by adding further oligonucleotides to the panel without significantly increasing complexity or cost.

For an ideal array, sequences should be selected as a probe and primer hybridized with only one target gene without cross-hybridization. The bacterial members of Enterobacteriaceae family have multiple similar *23S rDNA* sequences which makes it difficult to differentiate them. Therefore, in order to design additional oligonucleotides to identify species of this group of organisms, more strains should be studied (7, 16). In order to distinguish *Salmonella* spp., and *E. coli*, several probes were applied. However, the specific probe for *E. coli* had cross reaction with *Salmonella* spp., but it did not affect the discrimination because of using more than one probe to detect *E. coli* and *Salmonella* spp. In order to determine the ability of this method to detect several bacteria simultaneously, three mixtures of bacteria were combined together and examined with specific probes. The results indicated that each bacterium reacts with its strain specific probes. Using fluorescent labeled probes in microarray technique are not used as a routine approach in clinical laboratories for bacterial identification. In such methods, complex technical experiments such as regular stringency over the array chip, data analysis, and high cost are needed (28). Hence in the current study DIG-labeled primers were used to evaluate the hybridization reaction by naked eyes or conventional scanners. Wang et al. (29)

developed a membrane-array method to detect human intestinal bacteria by DIG-labeled primers without expensive microarray-arrayer and laser-scanner. They concluded that the method shares the same principle with microarray method, but does not need any expensive arrayer and scanner. DIG-labeled probes are not as expensive as fluorescence-labeled probes. Furthermore, the procedure is non-radioactive and can therefore be performed in any routine laboratory (28). For this reason, authors believe that diagnosis laboratories can easily perform this rapid and accurate test. The present study investigated an efficient tool for rapid, accurate and specific detection of four bacterial pathogens, and the results showed unique hybridization in combination with the oligonucleotide probes. In conclusion, the employed oligonucleotide microarray method was reliable and accurate to detect the four food-borne bacteria under study. Regarding the performance, the technique appears to be comparable with other reported methods, but is less expensive.

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Footnotes

Authors' Contribution: Meysam Sarshar and Nader Shahrokhi: Designing the study, obtaining samples and writing the manuscript; Reza Ranjbar: Drafting the manuscript; Caterina Mammina: Making substantial contributions to the conception of the study and interpretation of the data. All authors read and approved the final manuscript.

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